Volume-activated chloride currents in interstitial cells of Cajal

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Park, Sung Jin, Catherine M. Mckay, Yaohui Zhu, and Jan D. Huizinga. Volume-activated chloride currents in interstitial cells of cajal. Am J Physiol Gastrointest Liver Physiol 289: G791–G797, 2005; doi:10.1152/ajpgi.00050.2005.—Interstitial cells of Cajal (ICC) undergo marked morphological changes on contraction of the musculature, making it essential to understand properties of mechanosensitive ion channels. The whole cell patch-clamp technique was used to identify and to characterize volume-activated Cl− currents in ICC cultured through the explant technique. Hypotonic solutions (∼210 mosM) activated an outwardly rectifying current, which reversed near the equilibrium potential for Cl−. Time-dependent inactivation occurred only at pulse potentials of +80 mV, with a time constant of 478 ± 82 ms. The degree of outward rectification was calculated using a rectification index, the ratio between the slope conductances of inward rectification and outward rectification. The chloride channel blockers, DIDS and 5-nitro-2-(3-phenylpropyl-amino)benzoic acid, caused a voltage-dependent block of the outwardly rectifying Cl− current, inhibition occurring primarily at depolarized potentials. On exposure to hypotonic solution, the slope conductance significantly increased at the resting membrane potential (−70 mV) from 1.2 ± 0.2 to 2.0 ± 0.4 nS and at the slow-wave plateau potential (−35 mV) from 2.1 ± 0.3 to 5.0 ± 1.0 nS. The current was constitutively active in ICC and contributed to the resting membrane potential and excitability at the slow-wave plateau. In conclusion, swelling or volume change will depolarize ICC through activation of outwardly rectifying chloride channels, thereby increasing cell excitability.

METHODS

Cell preparation. Whole cell currents were measured from single ICC situated close to explants. The jejunum (∼1.5 cm long) was removed from newborn, 2-to 3-day-old CD-1 mice. By using sharp dissection, the mucosa and submucosa were removed by cleaving at the deep muscular plexus, leaving most ICC associated with the deep muscular plexus (ICC-DMP) out of the preparation studied. Segments of 0.5 mm in width and length were dissected in culture medium M-199 (GIBCO) and placed onto collagen-coated glass coverslips in a culture dish. The culture medium contained 10% fetal bovine serum, glutamine, and penicillin (GIBCO). After 1 day, ICC and smooth muscle cells grew out of the explants. The explants maintained rhythmic contraction patterns, and individual ICC even far from the explants could be seen to contract rhythmically. Cells were used 2–5 days after isolation; they were identified by the shape of the cell body, which was triangular or stellate, as well as their processes, which were thick compared with nerve processes. During this period of short-term culture, the ICC were spontaneously contractile and showed the typical rhythmic electrical pacemaker activity demonstrated previously (8, 21). In previous studies, we have demonstrated that there is 100% consistency between ICC identified in this manner and c-kit positivity, although all c-kit-positive cells do not necessarily have this exact shape.

Electrophysiology. Standard patch-clamp techniques were used to record whole cell membrane currents. Patch pipettes were pulled on a Sutter micropipette puller (P-87) from thin-walled, glass capillary tubing with filament and had resistances of 3–5 MΩ. Data were acquired using an EPC-9 amplifier (HEKA Elektronik) controlled by a PC running EPC Instramsoft software (Pulse/PulseFit) at 1 kHz (ramp pulse) or 10 kHz (step pulse) and filtered at 2.9 kHz with an analog 4-pole filter. Capacitance and series resistance (Rs) compensation was performed with the EPC-9 amplifier. Rs was compensated up to 80%. Cells were visualized with an inverted microscope (Zeiss, Axiowert 25). The recording chamber was constantly perfused with the extracellular solution at a rate of 1 ml/min. A perfusion device (Valvelink 8) was used to apply test solutions directly to cells. Grounding of the recording chamber was performed via an agar salt bridge (3% agar, 1 M KCl) minimizing liquid-junction potentials produced by test solutions. Reversal potentials were determined from current-voltage relationships obtained using voltage ramps from −80 to +80 mV (0.53 V/s). The cells were continuously bathed with extracellular solution (ECS) at room temperature (23–25°C). ECS contained the following (in mM): 135 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 dextrose (pH 7.35–7.40 with NaOH, ∼300 mosM). ECS contained the following (in mM): 130 Cs-aspartate, 10 NaCl, 4.5 magnesium ATP, 0.1 GTP, 10 EGTA, and 5 HEPES (pH 7.25–7.30).

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with CsOH). To control the osmolarity of the extracellular solutions, mannitol was used without the change of the concentration of the extracellular NaCl. The compositions of the isotonic (300 mosM) and various hypotonic (210 mosM) extracellular solutions are presented in Table 1. Osmolarity was tested with a vapor pressure osmometer (Advanced instruments, Micro Osmometer model 3300).

The test drugs, DIDS and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), were obtained from Sigma. For stock solutions (0.1 M) of DIDS and NPPB, external solution and ethanol were used, respectively.

Analysis. Data were analyzed using Origin software (version 6.0; Microcal Software, Northhampton, MA). Data were expressed as a means ± SE; n stands for number of observations. Student’s unpaired t-test was used to compare differences between two groups of data. Data were considered significant at \( P < 0.05. \)

The relative permeability of halide ions to chloride for whole cell currents under hypotonic conditions (\( P_{X/PCl} \)) was calculated from shifts in reversal potential (\( \Delta E_{\text{rev}} \)) with the constant field equation as follows:

\[
P_{X/PCl} = \frac{[Cl^-]_o \exp[\Delta E_{\text{rev}} (zF/RT)]/[x^-]_o}{\Delta E_{\text{rev}}}
\]

where \( \Delta E_{\text{rev}} \) is the shift in reversal potential seen from switching the bathing solution from the initial extracellular Cl\(^-\) concentration \([Cl^-]_o\) to a solution with an extracellular \( x^- \) concentration \([x^-]_o\). \( zF/RT \) was taken as 0.039 at 25°C, where \( z \) is charge, \( F \) is Faraday constants, \( R \) is gas content, and \( T \) is absolute temperature.

To analyze ion-channel rectification, the first derivatives of the polynomial functions (3rd order) obtained by curve fitting of current-voltage relationships were used to generate slope conductance (\( G_{\text{slope}} \)) vs. voltage relationships. A rectification index (RI) was calculated as the ratio between the slope conductances at \( 65 \) mV and \( 55 \) mV (\( G_{\text{slope}, 65 \text{ mV}} / G_{\text{slope}, 55 \text{ mV}} \)) (14).

**RESULTS**

**Volume-activated whole cell membrane currents in ICC.** Cells were voltage clamped in the whole cell configuration and held at \(-60\) mV. Cells were patched in control solution, and then isotonic solution was applied followed by hypotonic solution. Ramp pulses were applied from \(-80\) to \(80\) mV every \(10\) s, and the magnitudes of evoked currents at \(-80\) and \(80\) mV were plotted (Fig. 1A). ICC exposed to hypotonic solutions developed currents within 100–200 s that were reversible on return back to isotonic solutions. The current amplitude was \(104.3 \pm 13.0\) pA at \(-80\) mV and \(172.8 \pm 47.1\) pA at \(80\) mV (\(n = 12\)) under isotonic conditions. Exposure to hypotonic solution increased the current at \(-80\) mV to \(153.3 \pm 15.5\) pA.

**Table 1. Extracellular solutions**

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<td>Na-Asp</td>
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<td>KCl</td>
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<td>NMDG-Cl</td>
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<td>CaCl(_2)</td>
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<td>MgCl(_2)</td>
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<td>Mannitol</td>
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Concentrations are given in mM. pH was adjusted to 7.35–7.40 in all solutions.

Fig. 1. Exposure to hypotonic solutions activates a Cl\(^-\) current. A: time course of hypotonic solution-activated whole cell current at \(-80\) (square) and \(80\) mV (circle). Data were taken from currents generated in response to a ramp protocol from \(-80\) mV ascending to \(80\) mV. The graph is based on measurements taken every \(10\) s. Horizontal bars indicate the duration of the exposure to the various solutions in this and subsequent figures. B: average current amplitudes of control currents and currents obtained \(200\) s after switching to a hypotonic solution. Currents were measured at \(+80\) and \(-80\) mV. C: current responses to voltage ramps before, during, and after washout of a hypotonic solution at time points a-c shown in A. D: volume-activated current profile (b-a). \(V_h\), holding potential.
and at 80 mV to 952.9 ± 122.2 pA (n = 12). Comparison of whole cell current amplitudes under isotonic and hypotonic solution is shown in Fig. 1B. The reversal potential of the volume-activated (difference) current was −44.5 ± 3.7 mV (n = 12), not far from the Eq-Cl of −52.1 mV. The Eq-NSC and Eq-Na were −19.7 and 48.1 mV, respectively, in this experimental condition. The volume-activated currents displayed marked outward rectification (Fig. 1B). The degree of outward rectification was quantified as a rectification index (RI), the ratio between the slope conductances of 65 and 55 mV (Fig. 3C). The RI was 13.9 ± 1.5 with 76 mM [Cl]o (n = 5). In addition to the ramp protocols, step voltage pulses from −80 to 80 mV were given with increments of 20 mV to study time-dependent properties. Hypotonic solutions evoked currents that were reversible on return back to isotonic conditions (Fig. 2, A-C). Current-voltage relationships showed a reversal potential of −43.5 ± 4.5 mV (n = 7; Fig. 2E). Time-dependent inactivation occurred only at pulse potentials of +80 mV (Fig. 2D). The time course of inactivation at 80 mV was well fitted by a single exponential. Time constant (τ) was 478.1 ± 182.1 ms (n = 7; Fig. 2F). Fitting was not successful using second-order exponential fitting.

**Ionic basis of the volume-activated current in ICC.** Reduction of the extracellular sodium concentration ([Na+]o) did not result in a change in reversal potential of the volume-activated currents. The ΔE_{rev} was 2.3 ± 1.4 mV (n = 4) when reducing the [Na+]o from 65 to 0 mM (data not shown). However, the volume-activated current decreased on reduction of the extracellular chloride concentration ([Cl]o) from 76 to 11 mM (Fig. 3, A and B) and caused a shift in the reversal potential with an ΔE_{rev} of 39.4 ± 4.1 mV (n = 5; ΔE_{Cl} = 49.7 mV). The RI was 8.6 ± 2.4 with 11 mM [Cl]o (n = 5). The relative anion permeability of the volume-activated current was quantified on the basis of the shifts in reversal potentials. Replacement of Cl− in extracellular hypotonic solution with equimolar concentrations of iodine (I−), or aspartate (Asp) shifted the reversal potential of the whole cell currents. The reversal potential change (ΔE_{rev}) was −9.5 ± 1.2 mV for I− (n = 7).
and 13.7 ± 2.5 mV for Asp (n = 6). The relative permeability of I⁻ (P_I/P_Cl) and Asp⁻ (P_Asp/P_Cl) to Cl⁻ with the Goldman-Hodgkin-Katz equation was calculated at 1.4 ± 0.1 (n = 7) and 0.6 ± 0.1 (n = 6), respectively.

**Pharmacology of the volume-activated Cl⁻ current.** We investigated the effects of the conventional chloride channel blockers, DIDS and NPPB. Current-voltage relationships from cells exposed to hypotonic solution before and after the application of 100 μM DIDS revealed that DIDS blocked the volume-activated Cl⁻ current at −80 mV by 2.2 ± 1.4% and at +80 mV by 66.5 ± 0.9% (n = 4; Fig. 4, A and C). NPPB (100 μM) also blocked the volume-activated Cl⁻ current at −80 mV by 16.2 ± 11.7% and at +80 mV by 55.4 ± 0.4% (n = 4; Fig. 4, B and D). The volume-activated Cl⁻ current at +80 mV (1482.3 ± 123.3 pA) was significantly reduced within 1 min of exposure to DIDS (494.4 ± 28.8 pA, n = 4); however, at −80 mV (−371 ± 58.2 pA), much less current was affected by DIDS (−365.5 ± 51.1 pA, n = 4). Similar to DIDS, NPPB significantly decreased the outward current at +80 mV from 695.1 ± 21.9 to 310.3 ± 11.9 pA (n = 4) and at −80 mV from −217.6 ± 66.3 to −174.6 ± 29.8 pA (n = 4). These data indicate that the inhibition of the volume-activated Cl⁻ current by DIDS and NPPB was strongly voltage dependent. The effects of NPPB and DIDS were completely reversible. After washout of the drugs, the hypotonic solution brought back the normal increase in current (data not shown).

The outwardly rectifying Cl⁻ current under isotonic conditions. The majority of cells did not display significant changes in currents during the perfusion of isotonic solution. However, in 3 of 21 cells, outward currents did develop. Figure 5A shows a representative experiment in which the outward current increased and was subsequently reduced by 100 μM DIDS. The current amplitude was −36.1 ± 4.2 pA at −80 mV and 209.5 ± 13.9 pA at 80 mV (n = 3) after the application of isotonic solution. With a short time lag (<1 min), current increased to −46.7 ± 6.2 pA at −80 mV and to 351.5 ± 59.2 pA at 80 mV (n = 3). The E_{rev} of the activated-current was −44.6 ± 4.9 mV (n = 3), close to the Eq-Cl of −52.1 mV. The current showed outward rectification (Fig. 5, C and D).

The slope conductance increased with the activation of volume-activated Cl⁻ current. On exposure to hypotonic solution, the current increased at the resting membrane potential and at the slow-wave plateau potential. The slope of the current-voltage relationship was steeper under hypotonic conditions at both potentials (Fig. 6, A and B). To quantify the changes occurring in response to the activation of I_{Cl,vol} at the resting membrane potential and slow-wave plateau potential, the slope conductance both under isotonic and hypotonic conditions was calculated (Fig. 6C). On exposure to hypotonic solution, the slope conductance significantly increased at the resting membrane potential (−70 mV) from 1.2 ± 0.2 to 2.0 ± 0.4 nS (n = 9, P < 0.05) and at the slow-wave plateau potential (−35 mV) from 2.1 ± 0.3 to 5.0 ± 1.0 nS (n = 9, P < 0.05). Hence, the chloride conductance contributed to the resting membrane potential and influenced excitability at the plateau where action potential generation occurs.

**DISCUSSION**

This is the first report on volume-activated Cl⁻ currents in ICC. We observed that cell swelling by exposure to hypotonic solution activated an outwardly rectifying current. This current should be classified as a chloride current based on the fact that the current reversed direction close to the expected chloride equilibrium potential and was sensitive to the blocking action of the chloride channel inhibitors, NPPB and DIDS. Therefore, the current can be identified as a volume-activated Cl⁻ current (I_{Cl,vol}). I_{Cl,vol} in ICC has various characteristics that are similar to those reported for I_{Cl,vol} in other cell types but that distinguish the Ca^{2+}-activated Cl⁻ current [I_{Cl(Ca)}] (6, 24, 25). I_{Cl,vol} in ICC activates slowly with a time of 100–200 s under Ca^{2+} buffering with 10 mM EGTA. However, I_{Cl(Ca)} activates rapidly within ~1 s, and it is not possible to activate I_{Cl(Ca)} when [Ca^{2+}]_i is buffered to <100 nM (1, 12). I_{Cl,vol} in ICC exhibited outward rectification and only minor and slow inac-
tivation at large positive potentials. Outward rectification and inactivation at positive potentials are thought to be intrinsic channel properties of volume-activated Cl−/H11002 channels (6, 16, 17). The large currents at depolarizing potentials may be due to an increase in the local concentration of chloride at the binding site of volume-activated Cl− channel, which promotes channel opening (19). The relatively slower channel inactivation observed at positive potentials was also observed in rat brain endothelial cells (29) and rabbit nonpigmented ciliary epithelial cells (24). Much faster inactivation at positive potentials has been reported in canine colonic smooth muscle (2) and human prostate cancer cells (25). This discrepancy in different

Fig. 4. Effect of chloride channel blockers on volume-activated current. Whole cell currents in response to voltage ramps, applied from −80 to +80 mV obtained in hypotonic + 100 μM DIDS (A) and hypotonic + 100 μM NPPB (B). C and D show average current amplitudes of the volume-activated current. Current was obtained at +80 and −80 mV before and after application of the Cl− channel blockers DIDS (100 μM; C; *P < 0.05) and NPPB (100 μM; D; *P < 0.05).

Fig. 5. Spontaneous development of the chloride current under isotonic conditions. A: time course of whole cell current activation at −80 and 80 mV. B: average current amplitudes of the whole cell current. Current was measured at +80 and −80 mV before and after application of DIDS (100 μM). C: current responses to voltage ramps (from the experiment in A at time points indicated with a-c). D: difference current (b-a).
Fig. 6. Cell swelling causes increase in slope conductance. A: current-voltage relationships generated in response to a ramp protocol from −80 mV ascending to 80 mV in both isotonic (solid line) and hypotonic (dotted line) conditions. B: current-voltage relationships in box in A enlarged. Arrows indicate −70 and −35 mV. C: slope conductances (Gslope) under isotonic and hypotonic conditions. The first derivatives of the polynomial functions obtained by curve fitting of current-voltage relationships were used to determine slope conductance. Significance of differences was *P < 0.05 (n = 9).

cell types might be explained by variations in the threshold potential for depolarization-induced inactivation (17). I_{Cl,vol} in ICC has a higher permeability for iodine than for chloride, a property common to other volume-activated Cl⁻ channels (9, 16). The I⁻ > Cl⁻ selectivity is consistent with properties of the CLC-3 channel; however, the molecular identity of the protein-mediating volume-activated Cl⁻ channel remains controversial (9). The relative permeability of I_{Cl,vol} for I⁻ over Cl⁻ (P_I/P_{Cl}) was 1.4 ± 0.1 (n = 7). In comparison, I_{Cl(Ca)} has a high anion selectivity, and P_I/P_{Cl} is 3.5 (12). The pharmacological properties of the volume-activated Cl⁻ channel in ICC are similar to those previously reported in other cells (24, 25, 29). DIDS and NPPB blocked volume-activated Cl⁻ currents in a voltage-dependent manner; inhibition was much stronger at positive potentials. This voltage dependent block is believed to represent an interaction of the blockers with a site within the conducting pore of the ion channel, suggesting that the binding site of the blockers lies within the transmembrane electric field (9).

The ICC that were studied here are selected based on shape and the presence of contractile activity. Hence spontaneous contractile activity may activate the volume-activated Cl⁻ channel, although in most cells, contractile activity is diminishing quickly once the cell is placed at room temperature under the microscope. Nevertheless, we did observe the activation of chloride currents under isotonic conditions. These currents were identical to the volume-activated Cl⁻ current, because they shared the same pharmacology and similar outward-rectifying current profiles. Ramson and co-workers (20) studied the spontaneous activation of volume-activated Cl⁻ channel in human glioma cells.

Volume-activated Cl⁻ currents are involved in a variety of cellular functions, such as the maintenance of a constant cell volume, pH regulation, and control of membrane potential (15, 16). We previously reported that chloride channels play an important role in the generation of slow-wave activity in ICC. Rhythmic activation of chloride channels was associated with membrane potential changes suggesting a role in the initiation of slow waves (8, 30). Others have reported that the generation of the slow-wave plateau is likely mediated, to a large extent, by chloride channels (7). Furthermore, hyperpolarization of ICC induced by inhibitory enteric nerves may occur through inhibition of chloride channels (26). Conclusive evidence for the molecular identity of the chloride channels is not available yet; hence it is not known whether any of the above channels are identical to the volume-activated chloride channel described here. However, it is possible that I_{Cl,vol} may contribute to the excitability of the ICC because the slope conductance significantly increased at −70 mV from 1.2 to 2.0 nS and at −35 mV from 2.1 to 5.0 nS.

Sensing of mechanical activity is a complex process that involves mechanosensitive nerves (10, 11), mechanosensitive smooth muscle cells (13), and mechanosensitive ICC. Lyford and Farrugia (13) recently reviewed the mechanosensitive ion channels in smooth muscle cells, among them L-type calcium channels and Na⁺ channels. The mechanosensitive nature of L-type calcium channels in ICC has not been studied yet. Mechanically evoked responses in ICC will likely influence smooth muscle cells and enteric nerves to which the ICC are intimately coupled. In addition, other intracellular activities such as changes in nitric oxide or carbon monoxide could function as agents for communication between ICC and enteric nerve structures (3, 5, 23). Together these evoked activities may provide the smooth muscle cells and/or the enteric nervous system with the information needed to initiate distention-induced motor patterns.
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